

PMQR determinants among clinical isolates of ESBL and Amp C producing *Serratia marcescens* in Mansoura University Hospitals: A 6-year study

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Abstract

Background: Plasmid mediated quinolone resistant determinants (PMQRs) are found to be of clinical relevance because it causes decreased susceptibility to quinolones which are valuable antibiotics against intrinsically resistant *Serratia marcescens*. This study aimed to determine the occurrence of PMQR determinants (*qnr*, *aac(6')-Ib-cr* and *qep*) and their relation with extended-spectrum β -lactamase (ESBL) and / or Amp C- producing *S. marcescens*.

Material and Method: One hundred and eleven *S. marcescens* were tested for resistance to five quinolones by disc diffusion method. Testing for *qnr*, *aac(6')-Ib-cr* and *qep* was carried out by multiplex PCR, while ESBL and Amp C production were evaluated by double disc synergy test and cefoxitin Hodge test, respectively.

Results: A total of 51/111 (45.9%) *S. marcescens* were resistant to one or more of the tested quinolones. Among *S. marcescens* isolates, 13 (11.7%) were ESBL producers and 5 (4.5%) were Amp C producers. Multiplex PCR confirmed the presence of PMQR determinant in 14 (12.6%) of *S. marcescens* isolates and these were positive for *qnrA* and *qnrB* in 3 and 10 of the isolates, respectively, whereas one isolate co- harbored *aac(6')-Ib-cr* and *qnrA*. Among 13 ESBL producers, 3 and 5 isolates were positive for *qnrA* and *qnrB*, respectively (8/13; 61.5%), whereas one isolate which has co-expressed *aac(6')-Ib-cr* and *qnrA* was associated with Amp C production (1/5; 20%).

Conclusion: This study indicated that PMQRS play significant role in the occurrence and expansion of fluoroquinolone resistance in *S. marcescens*. They are also associated with ESBL and Amp C producers and important cause of infections in hospitalized patients.

Key words: PMQR, ESBL, Amp C, *Serratia marcescens*. Egypt

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Introduction

Serratia marcescens was considered in the past to be a harmless and non-pathogenic organism in humans, it is now becoming an important cause of hospital-associated infections including urinary tract infections, respiratory tract infections, septicemia, wound infections and meningitis [1].

Quinolones are powerful antibacterial agents against a wide range of bacteria species causing clinical infections [2]. They are particularly valuable against infections caused by Gram-negative bacteria species such as *S. marcescens* which is intractable to other antibiotics because of its intrinsic resistance [1].

Plasmid-mediates quinolone resistance (PMQR) involves three families, *qnrA*, *qnrB*, and *qnrS* [3]. The presence of these enzymes is of great importance because they are not merely able to confer resistance against most available quinolones but as well are often related to extended-spectrum β -lactamases (ESBLs) and/or plasmid-mediated Amp C β -lactamases (pAmp Cs) [4]. Six determinants of QnrA has been recorded worldwide from different enterobacterial species. This gene codes for a 218 amino acid protein of the pentapeptide family that prevent binding of quinolone to gyrase and topoisomerase IV protecting DNA [5]. Two other PMQR genes, called, *qnrB* and *qnrS*, have been recognized that code for QnrB and QnrS and share 41% and 60% amino acid homology with QnrA, respectively [6].

Another mechanism of conjugal quinolone resistance via an enzymatic inactivation of some quinolones by means of aminoglycoside acetyltransferase that codes for *cr* variant of *aac(6')-Ib* was detected. It acts by N-acetylation of their piperazinyl amine causing decreased susceptibility to quinolone. They have the same mechanism of action like QnrA [7].

Lastly, the extra PMQR determinants is the quinolone efflux pump gene, *qepA*. It shared a substantial resemblance to the major facilitator superfamily-type efflux pumps. This protein cause reduced susceptibility to many quinolones [8].

This study was conducted to find out the rate of PMQR determinants (*qnr*, *aac(6')-Ib-cr* and *qep*) in *S. marcescens* isolated from hospitalized patients at the Mansoura University Hospitals and their association with extended-spectrum β -lactamase (ESBL) and / or Amp C- producing *S. marcescens*.

Material and methods

Study design

This prospective study was carried out over a period of 6 years from January 2008 through January 2014, where *S. marcescens* strains were isolated from various clinical samples obtained from patients at the Mansoura University Hospitals (MUHs), Mansoura, Egypt. The samples processing were carried out in diagnostics and infection control unit (MDICU).

All samples were cultured on Blood and MacConkey's agar. Identification of *S. marcescens* isolates to species level was done primarily by Gram stain, colonial morphology, biochemical reactions including indole, methyl red, voges proskauer and citrate test (IMVC), motility on semisolid agar, DNAase, gelatin liquefaction tests and confirmed later with API 20E (bioMérieux) [9].

All isolates were tested for antibiotic susceptibility by disc diffusion method according to CLSI guidelines [10]. The isolates were tested against amoxicillin/clavulanic acid (20/10 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10µg), gentamicin (10µg), trimethoprim/sulfamethoxazole (25 µg), norfloxacin (10µg), nalidixic (30µg), ofloxacin (5 µg) ciprofloxacin(5 µg) and levofloxacin (5 µg) (Oxoid, UK). All isolates showed intermediate susceptibility were considered as resistant strains.

ESBL detection was detected by the use of a double-disk synergy test, and a test was considered positive if any distortion or zone increase towards the disc of amoxicillin-clavulanate was detected as reported by Paterson and Bonomo [11].

The cefoxitin Hodge test was used to screen strains producing Amp C β-lactamase and the test was interpreted positive in case of the presence

of clear growth of the control strain (ATCC *E. coli* 25922) along with the test isolate within the 22 mm inhibition zone after overnight Incubation at 37°C [12].

Multiplex PCR technique

Quick DNA extraction was done by a boiling technique that consists of a heating at 100°C of one colony in a volume of 100 µL of distilled water for 5 minutes then centrifugation of the cell suspension.

Multiplex PCR amplification of *qnrA*, *qnrB*, and *qnrS* was carried out according to Robicsek et al 2006. Primer sets used are listed in **table (1)** [13]. All primers were added to the template DNA and PCR master mix (Fermentas). The cycling conditions used were 94°C for 45 s (denaturation), 53°C for 45 s (annealing), and 72°C for 60 s (extension), which was repeated for 32 cycles. Another multiplex of PCR was held for amplification of *aac(6')-Ib* and *qep* with sequence of primers [14] mentioned as presented in Table (1). PCR conditions were 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s for 34 cycles. All positive PCR products for *aac(6')-Ib* were subsequently analyzed by digestion with *BtsCI* (Thermo Fisher

Table 1. Primers for amplification of PMQR molecular determinants

PMQR gene	sequence of primer 5' to 3'	size of amplicon (bp)	reference
<i>qnrA</i>	FW5'-ATTTCTCACGCCAGGATTTG-3' DW5'-GATCGGCAAAGGTTAGGTCA-3'	516	13
<i>qnrB</i>	FW5'-GATCGTGAAAGCCAGAAAGG-3' DW5'-ACGATGCCTGGTAGTTGTCC-3'	469	13
<i>qnrS</i>	FW5'-ACGACATTCGTCAACTGCAA-3' DW5'-TAAATTGGCACCTGTAGGC-3'	417	13
<i>aac(6')-Ib</i>	FW5'-TTGCGATGCTCTATGAGTGGCTA-3' DW5'-CTCGAATGCCTGGCGTGTTC-3'	482	14
<i>qep</i>	FW5'- AACTGCTTGAGCCCGTAGAT -3' DW 5- GTCTACGCCATGGACCTCAC-3'	596	14

Scientific™) to detect *aac(6')-Ib-cr*, that is deficient in the *BtsCI* restriction site found in the wild-type gene. The wild-type *aac(6')-Ib* PCR gave 210-bp and 272-bp fragments after restriction [15]. Amplification products were identified by their sizes in 2% ethidium bromide-stained agarose gels using 50bp DNA as control marker.

Statistical Analysis

Chi-square analysis was done to verify correlation between PMQR determinants and ESBL and Amp C producers. All statistical analyses were performed using Statistical Package for Social Science program (SPSS version 15.0 for windows, Chicago, IL). $P \leq 0.05$ was considered statistically significant.

Results

A total of 111 *S. marcescens* isolates were isolated from various clinical specimens. These isolates were obtained from the respiratory tract (69.6%), wounds (8.7%), blood (5.8%),

urine (2.9%), body fluids (3.9%), and the rest (9.1%) from other body sites. Resistance to one or more of the used quinolone was detected in 51/111(45.9%) of *S. marcescens* isolates. Among the isolated *S. marcescens*, 13 (11.7) isolates showed enhancement of the inhibitory zones of ceftazidime by clavulanic acid, signifying the existence of ESBL activity, while 5 had a positive results for amp C (4.5%) production using the cefoxitin Hodge test. No isolates showed combined ESBL and Amp C production. *S. marcescens* harbored *qnr* determinants in 14 isolates (12.6%). The *qnr* types, *qnrA* and *qnrB* genes were detected in 3 and 10 isolates, respectively, whereas one isolate co-expressed *aac(6')-Ib-cr* and *qnrA*. None of the isolates expressed *qep* gene. All detected PMQR gene determinants were quinolone resistant (14/51; 27.5%). Among ESBL producers, 3 and 5 isolates harbored *qnrA* and *qnrB* genes, respectively. While one isolate co- harbored *aac(6')-Ib-cr* and *qnrA* and was Amp C producers (1/5; 20%). The occurrence of PMQR genes was more frequent among ESBL *S. marcescens* (8 /13; 61.5%) than in non-ESBL producers (6/ 98; 6.1%) ($p < 0.05$).

Table 2. Distribution of PMQR among *S. marcescens* ESBL and non ESBL producers

PMQR genes	N. (%) <i>S. marcescens</i> ESBL producers non Ampc producers	N. (%) <i>S. marcescens</i> non ESBL producers	Total N. (%) <i>S. marcescens</i>
	13 (11.7)	98(88.3)	111 (100)
<i>qnrA</i>	3(37.5)	0 (0)	3 (2.7)
<i>qnrB</i>	5(62.5)	5 (5.1)	10(9)
<i>qnrS</i>	0 (0)	0(0)	0(0)
<i>qnrA+ aac(6')-Ib-cr</i>	0 (0)	1 (1)	1(0.9)
<i>qep</i>	0(0)	0 (0)	0(0)
Total PMQR	8 (61.5)	6 (6.1)	14 (12.6)

Table 3. Distribution of PMQR among *S. marcescens* Amp C and non Amp C producers

PMQR genes	<i>S. marcescens</i> Amp C producers	<i>S.marcescens</i> non Amp C producers	<i>S. marcescens</i>
	<u>N. (%)</u> 5 (4.5)	<u>N. (%)</u> 106 (95.5)	<u>N. (%)</u> 111 (100)
<i>qnrA</i>	0 (0)	3 (2.8)	3 (2.7)
<i>qnr B</i>	0(0)	10 (9.4)	10(9)
<i>qnr S</i>	0(0)	0(0)	0(0)
<i>qnrA+ aac(6')-Ib-cr</i>	1(20)	0 (0)	1(0.9)
<i>qep</i>	0(0)	0 (0)	0(0)
Total no. (%)	1(20)	13(12.3)	14 (12.6)

Table 4. Frequency of Quinolone resistance and PMQR determinants along with their types among isolated *Serratia marcescens* over 6 years.

year	N. (%) Quinolone –R <i>S.marcescens</i>	N. (%) PMQR producing <i>S. marcescens</i>	N. (%) PMQR determinant <i>S. marcescens</i>	Total n. (%) <i>S.marcescens</i> / year
2008 – 2009	2 (18.2)	Null	-	11 (100)
2009 – 2010	5 (22.7)	Null	-	22 (100)
2010 – 2011	5 (31.3)	1 (6.3)	<i>qnr B</i>	16 (100)
2011 -2012	11 (45.8)	3 (12.5)	2 <i>qnr B</i> , 1 <i>qnr A</i>	24 (100)
2012- 2013	13 (65%)	4 (20)	4 <i>qnr B</i>	20 (100)
2013 – 2014	15 (83.3)	6 (33.3)	3 <i>qnr B</i> , 2 <i>qnr A</i> , 1 combined <i>qnr A</i> and <i>aac(6')-Ib-cr</i>	18 (100%)

(Table 2) No statistical significance was observed regarding PMQR among *S. marcescens* Amp C (1/ 5; 20%) and non Amp C producers (13/ 106, 12.3%) ($p > 0.05$). **(Table 3)** Over the six years study period the overall frequency of PMQR gene determinants together with quinolone resistance have progressively increased by time. **(Table 4)**.

Discussion

This study has detected quinolone resistance at a rate of 45.9%, whereas a study by Park *et al.* in USA [16], found a higher rate of resistance to quinolones (66.3%), and the study of Yang *et al.* in China [17], reported much lower results (10.3%). The overall frequency of PMQR genes among the *S. marcescens* isolates in our study was 12.6% which is similar to other recent study that carried in China [3], while the study of Park *et al.* [16], on the contrary has showed much lower frequency (2.4%).

The present study demonstrates that the quinolone resistance was increased over time together with a growing shift to PMQR molecular determinants. This increase may be attributed to performance and enhancement of selection pressure of resistant mutants especially with extensive use of quinolones over the study period. In addition, further increase in the magnitude of the problem could be supported by the hypothesis that PMQR encourage emergence of QRDR mutations in *gyrA* and *parC* associated with quinolone resistance [14,17].

This study shows that *qnrB* is the most dominant (71.4%) among PMQR genes, and it is similar to many other studies over the world [6,7,18-21]. Moreover, this gene was the most common among ESBL producers signifying the relation between this gene and other resistance genes as observed by other researchers [22-25]. In contrast to the current results, the data reported by Yang *et al.* [3], found that the *aac(6')-Ib-cr* gene was the most prevalent

among *S. marcescens* ESBL producers. Additionally, Robicsek *et al.* [7], found this gene to be the most common in *E. coli* and the reason of this finding could not be explained. However, a different geographic distribution could be suggested as a cause for this variation. It is noted that *aac(6')-Ib-cr* and *qnrA* were co-expressed in one of our isolates, This finding support other reported results where some plasmids can carry both *aac(6')-Ib-cr* and *qnrA* [14]. This association confers the superior level of resistance to quinolones than do each gene by itself, although other studies found no relation between *qnr* genes and *aac(6')-Ib-cr* gene [7,26].

The frequency of the PMQR determinants in ESBL producing *S. marcescens* isolated from hospitals in this study was 61.5%, while a study done in Korea has detected only 4.8% as *qnr*-positive among ESBL producing *S. marcescens* strains [16]. However, high incidence (71.4%) has also been found in China [3]. A low incidence of *qnr* genes was detected among ESBL producing isolates of *E. coli* and *Klebsiella spp.* in France and Canada (1.6% and 1%, respectively), whereas high frequency of the *qnr* genes was reported in Egypt (26.6%) among ESBL producing *E. coli* [26], and in Spain, this prevalence was found among ESBL producing *E. coli* and *Klebsiella* at 5% and 8%, respectively [27].

Although our study has not detected any statistically significant results regarding the presence of PMQR determinants in association with pAmpCs (20%), it is still important to report that PMQR genes are frequently linked to ESBLs and/or pAmpCs and they can be transferred by conjugation [3].

The high frequency of PMQR among ESBL and Amp C producers in this study could be attributed to the genetic linkage and horizontal transfer of these resistance genes on plasmids [4].

In conclusion, quinolones are important drugs used in the treatment of infections caused by *S. marcescens*. Therefore, the increased frequency of

PMQRs among this organism over 6-year signifies a real therapeutic problem. Moreover, the close association between PMQR, ESBLs and Amp C genes in this organism would cause failure of treatment. This study recommends continuous surveillance to detect and molecular characterization of resistant bacteria isolates in hospitalized patients.

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